

Toll-Like Receptor Expression and Induction of Type I and Type III Interferons in Primary Airway Epithelial Cells

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Interferons (IFNs) are a critical component of the first line of antiviral defense. The activation of Toll-like receptors (TLRs) expressed by dendritic cells triggers different signaling cascades that result in the production of large amounts of IFNs. However, the functional consequences of TLR activation and differential IFN production in specific cell populations other than antigen-presenting cells have not yet been fully elucidated. In this study, we investigated TLR expression and polarization in airway epithelial cells (AECs) and the consequences of TLR agonist stimulation for the production of type I (IFN- α/β) and type III (IFN- λ) IFNs. Our results show that the pattern of expression and polarization of all TLRs in primary AEC cultures mirrors that of the human airways *ex vivo* and is receptor specific. The antiviral TLRs (TLR3, TLR7, and TLR9) are mostly expressed on the apical cell surfaces of epithelial cells in the human trachea and in primary polarized AECs. Type III IFN is the predominant IFN produced by the airway epithelium, and TLR3 is the only TLR that mediates IFN production by AECs, while all TLR agonists tested are capable of inducing AEC activation and interleukin-8 production. In response to influenza virus infection, AECs can produce IFN- λ in an IFNAR- and STAT1-independent manner. Our results emphasize the importance of using primary well-differentiated AECs to study TLR and antiviral responses and provide further insight into the regulation of IFN production during the antiviral response of the lung epithelium.

Epithelial cells lining the airway represent the first barrier to the entry of respiratory viruses and are their main replication target. In addition to its function as a mechanical barrier and in gas exchange, the airway epithelium plays an important role in pathogen detection and is a source of cytokines and other inflammatory mediators that modulate immunity in the respiratory tract (1–7). Airway epithelial cells (AECs) express Toll-like receptor 1 (TLR1) to TLR6 and TLR9 (8–11), and their activation with TLR agonists has been shown to induce the production of several cytokines, chemokines, and antimicrobial peptides. It is worth noting that the majority of these studies have been done at the mRNA level and using continuous cell lines or nonpolarized primary cells as responders to stimulation. Morphology and differentiation are critical in determining infection and immunity of the airway epithelium. First, AECs cultured under air-liquid interface (ALI) differentiate into ciliated cells that are more resistant to virus infection and mount less exacerbated inflammatory responses (12). Second, mucin is a negative regulator of TLR signaling exclusively expressed on the apical surfaces of differentiated AECs (13). Third, multiple receptors and adhesion molecules have a polarized distribution in AECs, i.e., the alpha/beta interferon (IFN- α/β) receptor (IFNAR) is exclusively expressed on the basolateral surface (14). Thus, primary polarized AEC cultures provide a valuable system that is a better representation of the airway epithelial microenvironment *in vivo* than cell lines (15–17).

One of the major downstream products of TLR signaling is the IFN family (18). IFNs are a diverse group of cytokines characterized for inducing antiviral resistance, and there are three types (type I, type II, and type III) based on their biological effects, receptor usage, and structure. Only type I and type III IFNs are directly produced in response to virus infection. Type I IFNs are key immune regulators essential for mounting a robust immune response to many viral infections (19, 20). All subtypes of type I IFNs engage the ubiquitously expressed IFNAR and initiate a sig-

naling cascade that leads to the induction of >300 IFN-stimulated genes (21). Type III IFNs include interleukin-28A (IL-28A), IL-28B, and IL-29 (also known as IFN- λ 1, IFN- λ 2, and IFN- λ 3) (22, 23) and signal through the IFN- λ receptor (IFNLR) that is composed of an exclusive IFN- λ R1 chain and a shared IL-10R2 chain (23). Despite the low amino acid homology between type I and type III IFNs, they trigger common signaling pathways and biological activities (24, 25). This functional redundancy is contested by the different receptor distributions and by the differential regulation of type I and type III IFN production during infection. Although IFNAR is present in all cells, the expression of IFNLR is limited to epithelial cells (26, 27). Type III IFNs are produced at higher levels and during longer times in the lung than type I IFNs during influenza virus infection (28). These differences are likely to result in cell- and tissue-specific effects of type I and type III IFNs during antiviral responses.

In the present study, we aimed to get a better understanding of the role of TLRs in the production of IFNs by AECs. We used human primary polarized AEC cultures to assess the expression of TLRs compared to that of human trachea and examined the induction of IFNs after activation with different TLR ligands and during influenza virus infection. We found differential and receptor-specific TLR expression on ciliated/basal cells or on the apical/basolateral cell membrane of the airway epithelium. Our data show that type III IFN is the predominant IFN produced by the airway epithelium and that TLR3 is, among the different TLR

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ligands evaluated, the only inducer of IFN production by AECs. The present study also sheds light on the complexity of IFN- λ regulation and emphasizes the importance of using primary well-differentiated AECs to study the antiviral response of the lung epithelium.

MATERIALS AND METHODS

Primary human and mouse AECs. Human trachea sections were obtained from deidentified histology specimens banked in the Anatomic Pathology Laboratory at Nationwide Children's Hospital. Human airway tracheobronchial epithelial cells from non-cystic fibrosis patients were obtained from airway specimens resected at lung transplantation according to Nationwide Children's Hospital Institutional Review Board-approved protocols. Epithelial cells were removed from the mainstream bronchi by protease digestion and plastic adherence and plated at a density of 300,000 cells per well on permeable Transwell-Col supports. Human AEC cultures were maintained under ALI for 4 to 6 weeks to form well-differentiated, polarized cultures that resemble the *in vivo* pseudostratified mucociliary epithelium as previously described (29). Beas-2B cells were grown in bronchial epithelial cell basal medium. Wild-type BALB/c and C57/BL6 mice were purchased from Harlan Laboratories and The Jackson Laboratory, respectively. B6 TLR3^{-/-} mice (Jackson Laboratory), BALB/c IFNAR^{-/-} mice, and STAT1^{-/-} mice were bred in-house. All animals were maintained in BL2 containment under pathogen-free conditions. The Institutional Animal Care and Use Committee at The Research Institute at Nationwide Children's Hospital approved all of the animal studies described in this work. Tracheal epithelial cell isolation and culture was performed as previously described (29). Briefly, nonadherent tracheal cells were seeded onto collagen-coated polyester membranes (Corning-Costar) and, after reaching confluence, the cells were incubated under ALI for 10 to 14 days. Bone marrow-derived dendritic cells were grown in tissue culture medium supplemented with 20 ng of granulocyte-macrophage colony-stimulating factor (GM-CSF)/ml for 6 days as previously described (30).

Immunohistochemistry, flow cytometry, and antibodies. Antibodies against human TLRs were from Santa Cruz Biotechnology (TLR1 H-90, TLR2 H-175, TLR3 H-125, TLR4 H-80, TLR5 H-127, TLR6 H-90, TLR7 H-114, TLR8 H-114, TLR9 H-100, and TLR10 H-165). As secondary antibodies, we used Texas Red-conjugated goat anti-rabbit IgG H+L (Vector Laboratories) for immunohistochemistry and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Abcam) for fluorescence-activated cell sorting analysis. Mouse TLR3 was detected with phycoerythrin-conjugated anti-mouse TLR3 [IgG1(κ)] and its respective isotype control (both from Imgenex). Anti-TLR3 blocking antibody (InvivoGen) or rat IgG2 α isotype control (eBioscience) were used at 40 μ g/ml. Immunohistochemistry was performed on paraffin-embedded tissue sections, and analysis was performed with an Olympus BX61 microscope. For flow cytometry analysis, AEC cultures were treated with cold EDTA solution and mechanically disrupted to obtain a single cell suspension. Cells were blocked with Fc-block, stained with the mixture of the antibodies indicated above and with Blue-Dead-Cell-Stain (Invitrogen), and then fixed with paraformaldehyde. Only when indicated, the cells were permeabilized with saponin prior to incubation with antibodies to allow for intracellular staining. Flow cytometry data were acquired on a BD LSR II and analyzed using FlowJo software. Gates were set using negative controls and IgG isotype controls (BD Pharmingen). The results were expressed as the percentage or median fluorescence intensity (MFI) of the total live cell population after subtracting the MFI of control cells stained with the appropriate isotype control antibodies.

Viruses. Influenza A/WSN/33 (H1N1) and influenza A/Udorn/72 (H3N2) viruses were grown for 48 to 72 h in day-10-embryonating chicken eggs at 37.5°C, and virus titers were determined by an immunofluorescence focus assay. AEC cultures were exposed to 2×10^5 PFU of influenza Udorn or influenza WSN (multiplicity of infection of apical

cells of 1) or mock treated for 2 h, and the supernatants were harvested 8, 16, and 24 h later.

TLR agonists, stimulations, and protein analysis. The following TLR ligands (InvivoGen) and concentrations were used for the stimulation of primary human and mouse AECs, human bronchial Beas-2B cells, GM-CSF-derived mouse dendritic cells grown as indicated (31), and murine alveolar macrophage MH-S cells: synthetic triacylated lipoprotein (Pam3CSK4; 1 μ g/ml), high-molecular-weight poly(I-C) (10 μ g/ml), lipopolysaccharide (LPS) from *E. coli* O111:B4 (10 μ g/ml), recombinant flagellin from *Salmonella enterica* serovar Typhimurium (RecFlast; 1 μ g/ml), a thiazoloquinolone derivative (CL075; 5 μ g/ml), and CpG ODN (5 μ g/ml) (all from InvivoGen). Optimal agonist concentrations and time of analysis were based on manufactured recommendations and titrated in dose-response assays. The cells were treated with the indicated TLR agonist or mock treated for 2 h, washed, and incubated with culture medium at different times. Anti-TLR3 blocking antibody (InvivoGen) or rat IgG2 α isotype control (eBioscience) were used at 40 μ g/ml. Bafilomycin A1 (Sigma) was used at 500 nM. The cell culture supernatants from human cells were assayed with IFN- α multi-subtype and IFN- β enzyme-linked immunosorbent assay (ELISA) kits (PBL) and with IL-6, IL-8, and IL-29 ELISA kit (eBioscience) according to the manufacturer's instructions. The cell culture supernatants derived from murine cells were analyzed by using a standard IFN- $\alpha\beta$ bioassay (30) and with IFN- β and IL-28 ELISA kit (eBioscience) according to the manufacturer's instructions.

Statistical analysis. ELISA results were analyzed using Prism (version 5.0; GraphPad). The experiments were repeated two to four times, and a representative experiment is presented in which triplicate well replicates are expressed as means \pm the standard deviation (SD). A Student *t* test was used for analysis of data, where $P \leq 0.05$ was considered statistically significant.

RESULTS

TLR expression and polarization in human tracheal epithelial cells and primary hAEC cultures. TLRs play prominent roles in initiating immune responses, but their specific roles in particular cell populations are not fully recognized. To begin understanding how the airway epithelium may recognize pathogens and exogenous antigens, we assessed TLR1-TLR10 protein expression and distribution in the epithelium of the human trachea (Fig. 1). TLR1 and TLR3 were detected on the surfaces and in the cytoplasm of epithelial cells throughout the whole width of the pseudostratified epithelium. TLR1 expression was more conspicuous on the luminal surface of the tracheal epithelium. TLR3 was mainly expressed on the luminal and basal mucosal surface. TLR2 and TLR6 showed a predominant basolateral distribution. Although TLR2 was weakly expressed, TLR6 exhibited an intense basolateral staining. TLR4, TLR5, TLR7, TLR9, and TLR10 were weakly expressed in the luminal surface of the tracheal mucosa (terminal plates and cilia). We were unable to detect any TLR8 expression at protein level. It is interesting to note that TLR3, TLR7, and TLR9, traditionally expressed in the endosomal compartments of dendritic cells (32), were expressed both intracellularly and on the cell surfaces of AECs. Our results show that each TLR has a unique pattern of expression and distribution on tracheal epithelial cells that is likely to contribute to the regulation of the innate response of AECs to pathogenic and commensal microorganisms in the respiratory tract.

To study the response of the human airway epithelium to different TLR stimuli, we used a polarized model of human or mouse primary epithelial cell cultures (29). First, in order to validate our *in vitro* model, we examined the expression of TLRs in primary polarized hAECs (Fig. 2). TLR1 and TLR3 demonstrated both apical and basolateral expression on the cell surface and in the

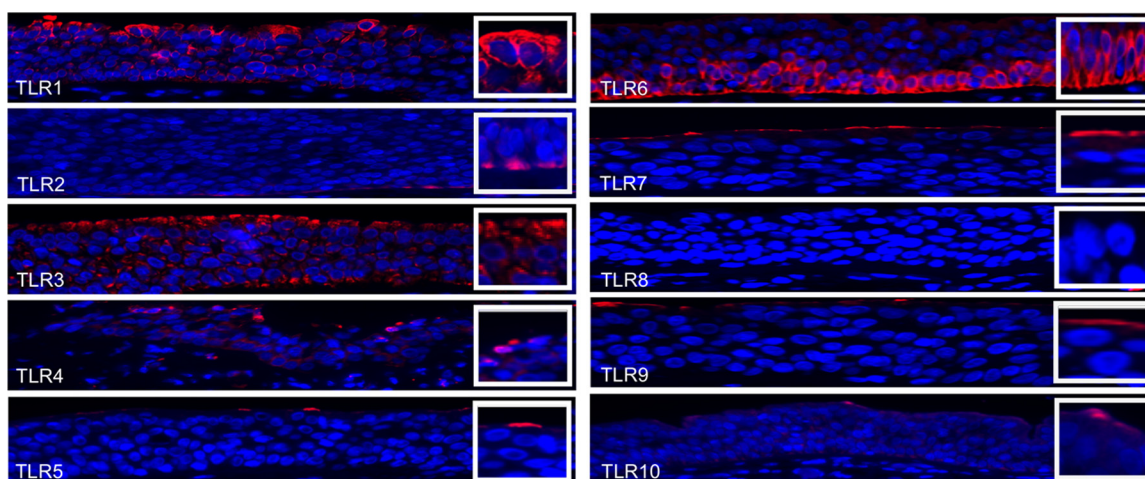


FIG 1 TLR expression in the airway epithelium of the human trachea. Immunofluorescence with anti-human TLR-specific antibodies was used to detect the expression and distribution of TLR1 to -10 in AECs in tissue sections of human tracheas. The lumen of the airways is on the top.

cytoplasm. TLR2 and TLR6 were predominantly localized in the basolateral surface with only some apical expression. Expression of TLR4, TLR5, TLR7, TLR9, and TLR10 was mainly observed on the surface of the apical cells, especially in the terminal bars or in the cilia. We did not detect TLR8 expression. In summary, the pattern of expression for all TLRs in hAEC cultures corroborated our findings from the trachea.

Next, we used cell surface flow cytometry staining to corroborate the cell surface localization of TLRs in primary mTECs and in the human bronchial cell line Beas-2B (Fig. 3). All TLRs analyzed were expressed in mTECs with a frequency of 4.8 to 16.4% positive cells and an MFI of 120 to 227 (TLR2 and TLR6, respectively). We detected reduced TLR8 expression in mTECs, whereas human trachea and primary hAECs were negative. As in human epithelial cells, TLR3, TLR7, and TLR9 were detected on the cell surfaces of mTECs. Beas-2B cells are frequently used as a surrogate model for hAECs. Our analysis showed that Beas-2B cells expressed all TLRs analyzed and that TLR1 was the predominant TLR expressed in Beas-2B cells.

IFN- λ is the predominant IFN produced by human airway epithelial cells (hAECs) after poly(I:C) or influenza virus stimulation. The airway epithelium produces IFNs in response to viral and bacterial pathogens (33–35). However, inflammation of the

airways is extremely detrimental for gas exchange, and the epithelium has an active role in regulating lung homeostasis. Here, we wanted to elucidate which TLRs were responsible for IFN production by AECs, and which types of IFN were induced by different stimuli. Thus, we incubated primary hAECs with various purified TLR agonists and measured the production of IFN- α , IFN- β , and IFN- λ at different times after stimulation (Fig. 4). The ligands used were Pam3CSK4 (TLR1/2 agonist), poly(I:C) (TLR3 agonist), LPS (TLR4 agonist), RecFlast (TLR5 agonist), CL075 (TLR7 agonist), or CpG (TLR9 agonist). Influenza virus was used as a positive control because it results in the activation of TLR3- and RIG-dependent production of cytokines (36). Influenza virus was the only stimulation that induced IFN- α , IFN- β , and IFN- λ production, albeit with different temporal kinetics and at different concentrations. IFN production in response to influenza virus stimulation followed a time-dependent pattern of secretion increasing from 8 to 24 h, although IFN- α could not be detected at 8 h. IFN- λ was the predominant IFN secreted in response to influenza virus (1,801 pg/ml at 24 h). Half this amount of IFN- β was measured at 24 h, whereas the amount of IFN- α at the same time point was 449 pg/ml. Of all of the TLR agonists tested, only poly(I:C) was able to induce a detectable IFN response. IFN- β and IFN- λ were readily detected at 8 h, and IFN- λ (919 pg/ml) was the

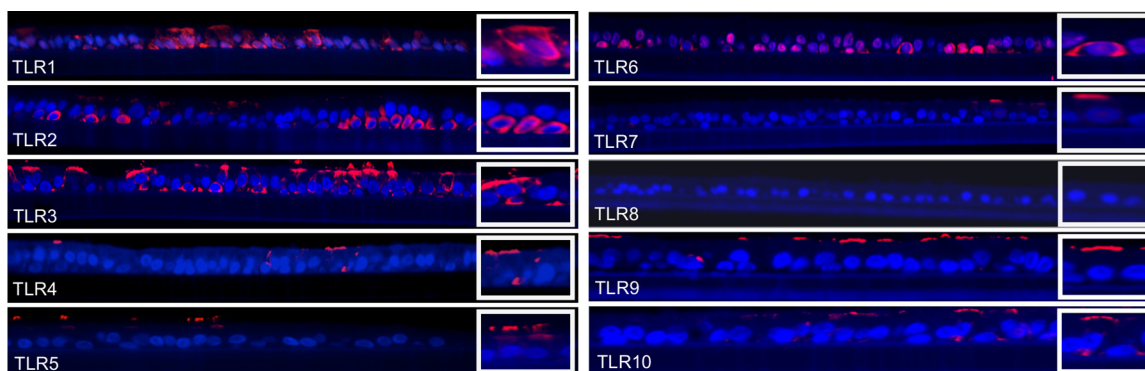


FIG 2 TLR expression in human primary AEC cultures. Immunofluorescence with anti-human TLR-specific antibodies was used to detect the expression and distribution of TLR1 to -10 in polarized hAEC cultures. The apical surface is on the top.

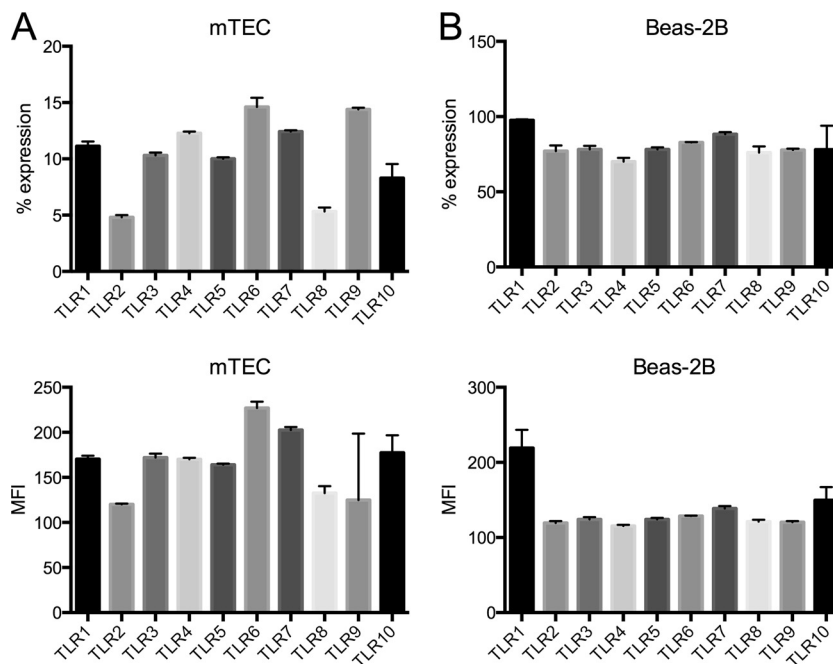


FIG 3 Cell surface TLR expression in mouse epithelial cells and in Beas-2B cells. Flow cytometry was used to detect the cell surface expression of TLR1 to -10. (A) mTECs; (B) Beas-2B cells. The percent expression is shown in the top panels, and the MFI is shown in the bottom panels. Isotype control and secondary antibody staining were used as controls. The data are representative of two independent experiments (means and SD).

predominant IFN secreted by primary hAECs upon poly(I-C) stimulation. The kinetics of IFN- β and IFN- λ production by hAECs in response to poly(I-C) stimulation were different. The production of IFN- β kept declining at 16 and 24 h (125 and 98 pg/ml, respectively), whereas the IFN- λ concentration increased over time (301 pg/ml at 8 h, 919 pg/ml at 16 h, and 1,484 pg/ml at 24 h). No IFN- α could be detected in response to poly(I-C) stimulation at any time point analyzed. Importantly, none of the other TLR agonists tested induced the secretion of any measurable amounts of IFN- α , IFN- β , or IFN- λ by hAECs. Altogether, these results indicate that IFN- λ is the predominant IFN produced by human airway epithelium after TLR signaling or influenza virus stimulation.

To elucidate whether the restricted production of IFNs observed in primary hAECs in response to TLR agonist stimulation was due to impaired TLR signaling, we measured the production of other cytokines such as IL-8 and IL-6. The results show that all of the stimulations tested induced robust production of IL-8, with poly(I-C), RecFlast, and influenza virus inducing the secretion of $>10^5$ pg of IL-8/ml (Fig. 4D). IL-6 production was induced by poly(I-C), RecFlast, and influenza virus stimulation (Fig. 4C). Altogether, these results indicate that although all of the TLR ligands tested were capable of inducing AEC activation and cytokine production, only poly(I-C) stimulation could trigger the production of IFNs.

Next, we wanted to corroborate whether the restricted IFN response observed in hAEC cultures could be extended to murine cells and continuous cell lines and whether it was distinct from that of antigen-presenting cells. Thus, we incubated mTECs and Beas-2B cells with several TLR ligands and influenza virus and assessed the production of type I IFN. Type I IFN bioactivity could only be detected in response to poly(I-C) or influenza virus stim-

ulation (Fig. 5), as previously observed with primary hAECs. To verify that this restricted type I IFN response was specific for AECs and not due to the stimulatory ligands used, we applied the same concentration of TLR ligands to primary mouse dendritic cells and to a cell line of alveolar macrophages (MH-S). Under similar experimental conditions, dendritic cells produced large amounts of type I IFN in response to stimulation with poly(I-C) (2,400 U/ml), LPS (1,200 U/ml), CL07 (5,300 U/ml), CPG (4,800 U/ml), and influenza virus (4,800 U/ml). MH-S alveolar macrophages also produced conspicuous amounts of type I IFN after stimulation with poly(I-C) (800 U/ml), LPS (400 U/ml), RecFlast (100 U/ml), CPG (50 U/ml), and influenza virus (1,600 U/ml). Altogether, the data show that dendritic cells and alveolar macrophages responded to a broader range of TLR ligands and produced greater amounts of type I IFN compared to AECs.

IFN- λ production is independent of PAMP synergy and occurs in the absence of IFN- α receptor signaling. Since AECs only secreted IFNs in response to poly(I-C) stimulation, we sought to determine whether simultaneous stimulation with different TLR agonists would enhance IFN production. Thus, we incubated Beas-2B cells with different combinations of TLR ligands and measured the IFN- λ production 24 h later. The data show that Beas-2B cells produced IFN- λ only when poly(I-C) was present in the mix of TLR agonists, and no significant differences in IFN- λ production could be detected between samples incubated only with poly(I-C) or with poly(I-C) plus a different TLR ligand (Fig. 6A). We next wanted to corroborate these observations with primary hAECs. We incubated hAEC cultures with selected combinations of TLR ligands and measured the IFN- λ and IL-8 production. The data show that hAECs produced IFN- λ only when poly(I-C) was present in the mix of TLR agonists and that increased IFN- λ production could not be detected when testing syn-

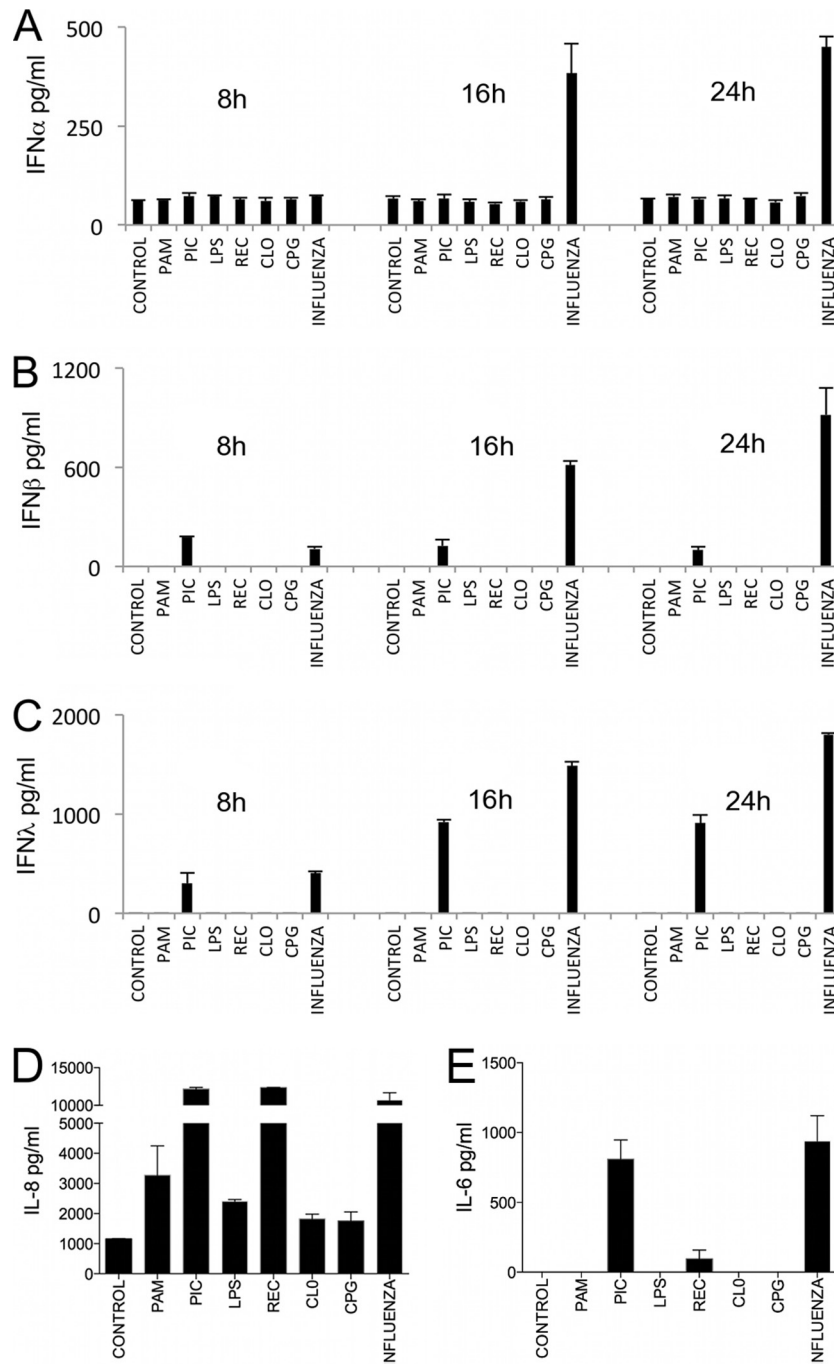


FIG 4 Kinetics of IFN production by human AECs in response to stimulation with PAMPs and influenza virus. (A) IFN- α ; (B) IFN- β ; (C) IFN- λ ; (D) IL-8 at 24 h; (E) IL-6 at 24 h. Primary well-differentiated hAECs were incubated with Pam3CSK4, poly(I-C), LPS, RecFlast, CL075, or CpG with 2×10^5 PFU influenza virus H3N2, as indicated in Materials and Methods. Culture supernatants were harvested at 8, 16, and 24 h and analyzed by ELISA. The data are representative of two independent experiments with three samples per group (means and SD).

ergic TLR ligands (Fig. 6B). A parallel analysis of IL-8 production showed that all combinations of TLR agonists tested induced hAEC activation and IL-8 secretion (Fig. 6C). We did not observe increased IL-8 production when poly(I-C) was added together with other TLR agonists (Pam3CSK4 and/or LPS). Nevertheless, Pam3CSK4 and LPS elicited higher production of IL-8 when added simultaneously (Fig. 6C), suggesting that other cytokines

different from IFN- λ respond to TLR synergy. Altogether, these data indicate that IFN- λ production by AECs is dependent on poly(I-C) stimulation and independent of TLR synergy.

To elucidate the impact of type I IFN signaling on IFN- λ production by AECs, we used mTEC cultures from mice lacking IFNAR, which is responsible for type I IFN signaling amplification, or from mice lacking STAT1, which is a key signaling component

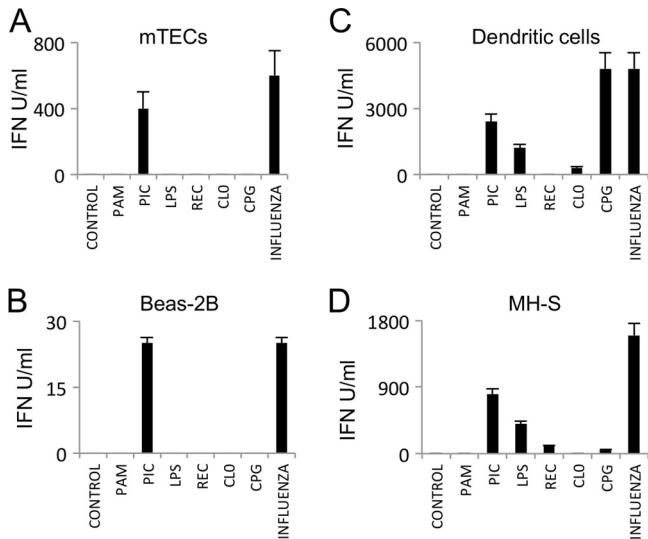


FIG 5 Type I IFN production by epithelial cells, dendritic cells, and alveolar macrophages. (A) mTECs; (B) Beas-2B cells; (C) bone marrow-derived mouse dendritic cells; (D) alveolar macrophage cell line MH-S. Cells were incubated with Pam3CSK4, poly(I-C), LPS, RecFlast, CL075, or CpG or with 2×10^5 PFU influenza virus, as indicated in Materials and Methods. The levels of type I IFN bioactivity were measured in culture supernatants at 24 h. The data are representative of two independent experiments with three samples per group (means and SD).

for the production of all IFNs. The data show that the amount of IFN- λ produced by IFNAR $^{-/-}$ mTECs in response to poly(I-C) stimulation was significantly lower than that of wild-type mTECs (38 and 158 pg/ml, respectively), but IFN- λ was still produced by IFNAR $^{-/-}$ cells (Fig. 7A). We could not detect any IFN- λ produc-

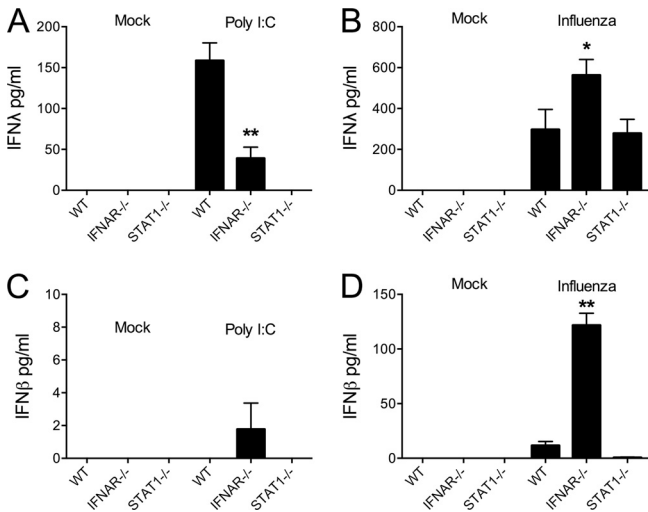


FIG 7 Regulation of IFN- β and IFN- λ production in mTECs. mTEC cultures were derived from wild-type, IFNAR $^{-/-}$, and Stat1 $^{-/-}$ mice. The cells were stimulated with poly(I-C) (left panels) and influenza virus (right panels) as described in Materials and Methods, and the concentrations of IFNs were measured by ELISA 24 h later. (A) IFN- λ in response to poly(I-C); (B) IFN- λ in response to influenza virus; (C) IFN- β in response to poly(I-C); (D) IFN- β in response to influenza virus. The data are representative of two independent experiments with three samples per group (means and SD). *, $P \leq 0.05$; **, $P \leq 0.005$.

tion after stimulation of STAT1 $^{-/-}$ mTECs with poly(I-C). These results support the concept that STAT1 activation by IFN- λ alone is sufficient to mediate autocrine type III IFN production in response to poly(I-C) stimulation. However, when exposed to influenza virus IFNAR $^{-/-}$ mTECs secreted more IFN- λ than wild-type

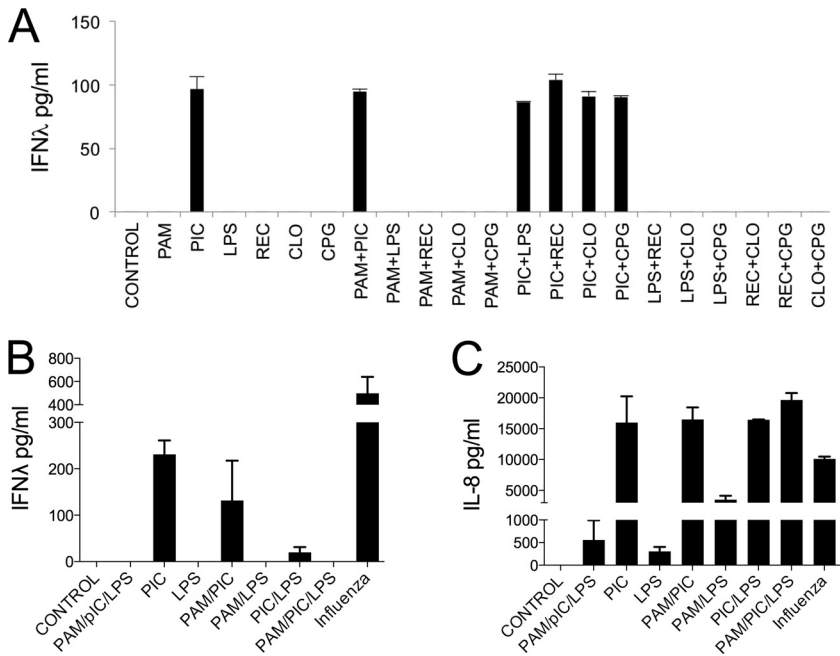


FIG 6 Role of synergistic TLR stimulation on the regulation of IFN- λ production in epithelial cells. Cells were stimulated with influenza virus, Pam3CSK4, poly(I-C), LPS, RecFlast, CL075, and CpG alone or in combination. The levels of IFN- λ and IL-8 were measured in culture supernatants at 24 h by ELISA. (A) IFN- λ production in Beas-2B cells; (B) IFN- λ production in primary hAECs; (C) IL-8 production in primary hAECs. The data are representative of two independent experiments with three samples per group (means and SD).

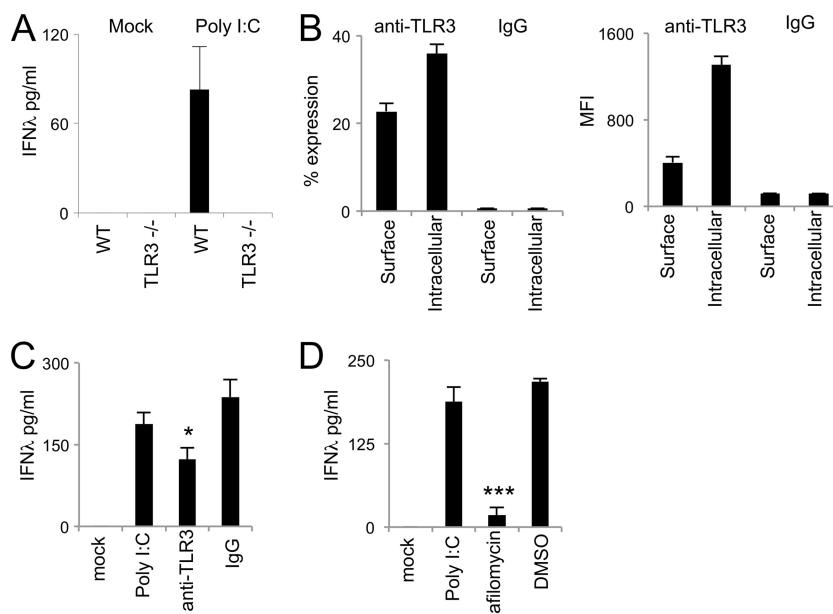


FIG 8 TLR3-poly(I-C) interaction mediates IFN- λ production by AECs. (A) IFN- λ production in wild-type and TLR3^{-/-} mTECs in response to poly(I-C) stimulation. (B) Intracellular and cell surface TLR3 expression in primary hAECs. The left panel shows the percent expression, and the right panel shows the MFI. Cells were stained with anti-TLR3 or with rat IgG2a isotype control antibodies. (C) Epithelial IFN- λ production in the presence of anti-TLR3 blocking antibodies. (D) Epithelial IFN- λ production in the presence of bafilomycin. IFN- λ was measured in culture supernatants by ELISA 24 h after stimulation. The data are representative of two independent experiments with 3 samples per group. Error bars indicate the SD. *, $P \leq 0.05$; ***, $P \leq 0.0005$.

mTECs, and STAT1^{-/-} mTECs produced similar amounts of IFN- λ than their wild-type counterparts (Fig. 7B). Altogether, these results demonstrate that IFNAR signaling is not essential for IFN- λ production in the airways epithelium and suggest the existence of a Stat1^{-/-} independent pathway of IFN- λ production in response to influenza virus infection. Simultaneous detection of IFN- β in mTECs corroborates our previous findings indicating that IFN- λ is the predominant IFN produced the epithelium of the airways after poly(I-C) or influenza virus stimulation (Fig. 7C and D).

TLR3-poly(I-C) interaction mediates IFN- λ production by AECs. Our previous findings indicate that poly(I-C) mediates robust IFN responses in AECs. To corroborate that this response is mediated by TLR3 and not by cytoplasmic sensors such as RIG-I/MDA-5 (37, 38) or NLRP3 (39), we stimulated mTEC cultures derived from wild-type and TLR3^{-/-} mice with poly(I-C). We observed that TLR3-deficient mTECs did not produce any IFN- λ in response to naked poly(I-C) stimulation, in contrast to wild-type mice (Fig. 8A). This result suggests that TLR3 and not cytoplasmic sensors account for the response to naked poly(I-C) stimulation of mTECs.

Next, we wanted to examine the role of cell surface TLR3 in the response of AECs to synthetic double-stranded RNA (dsRNA) stimulation. First, we quantified the level of TLR3 cell surface expression on hAECs. We found that 25% of hAECs expressed TLR3 on their surface and that after intracellular staining the frequency of TLR3-positive cells would increase slightly to 36% (Fig. 8B). These data indicate that a large fraction of hAECs expressing TLR3 do so on their cell surfaces. Second, we analyzed the effect of blocking TLR3 signaling. As previously described for fibroblasts (40), TLR3 expressed on the cell surface of epithelial cells should be readily accessible to blocking antibodies, as opposed to endo-

somal TLR3. Thus, we treated epithelial cells with an anti-TLR3 neutralizing antibody and then stimulated the cultures with poly(I-C). The data show that TLR3 antibody blockade produced a statistically significant reduction of IFN- λ production, but it did not completely abrogate its production (Fig. 8C). This result suggested that both cell surface and endosomal TLR3 participate in dsRNA signaling in AECs. Endosomal acidification is a prerequisite for endosomal TLR activation in dendritic cells (41–43). However, we do not know whether cell surface TLR3 can signal after agonist binding or whether further internalization and endosomal acidification are needed for its activation in AECs. Beas-2B cells were stimulated with poly(I-C) in the presence or not of bafilomycin A1, an inhibitor of endosomal acidification. As shown in Fig. 7D, pretreatment with bafilomycin led to a significant reduction in the amount of IFN- λ produced. These results suggest that in spite of its cell surface expression, TLR3 internalization and endosomal acidification are fundamental steps for dsRNA-TLR3 signaling by the airway epithelia.

DISCUSSION

Our understanding of how the epithelium of the human airways responds to virus infection is still limited. Here, we used a polarized well-differentiated model of primary human and murine epithelial cell cultures that closely imitates the morphology of the respiratory mucosa in order to analyze the expression of TLRs and their IFN response to selected TLR agonists and influenza virus. Epithelial cell lines express TLRs and other PRRs, such as NODs, RIG-I, and MDA5 (8–11, 44–48). Our results shed light on the specific distribution of each TLR in the airway epithelium and demonstrate that the pattern of TLR expression and polarization is similar between human tracheal epithelial cells and primary hAEC cultures. TLR3 was expressed on the apical and basolateral

surfaces of AECs, and TLR1, TLR4, TLR5, TLR7, TLR9, and TLR10 were mostly expressed on the luminal cell surface. On the contrary, TLR2 and TLR6 had a prevalent basolateral distribution, which will prevent the interaction between PAMPs located in the airway and their respective receptors unless the integrity of the epithelial barrier is compromised. The expression of antiviral TLRs (TLR3, TLR7, and TLR9) mainly on the apical cell surfaces of AECs is fundamentally distinct from their “standard” localization in endosomal membranes of antigen-presenting cells (32) and contrast with the basolateral localization of TLR2 and TLR6 that heterodimerize together to recognize bacterial lipopeptides (49). These results suggest that AECs express distinct TLRs with a distribution and polarization that likely serves tissue-specific biological needs.

Among the TLR agonists analyzed in the present study, only poly(I-C) was capable of inducing the production of IFNs, including IFN- β and IFN- λ but not IFN- α , in primary hAECs. Since all TLRs use the adaptor protein MyD88 except TLR3, which uses TRIF, our findings suggest that TLR-mediated IFN- λ induction in AEC is mediated by TRIF and not by MyD88. Poly(I-C), a synthetic analog of dsRNA, is a potent inducer of IFN- α in dendritic cells (50), and it has also been shown to induce IFN- β in primary SAEC (51) and in Beas-2B cells (52). Our observations highlight the existence of differential temporal and quantitative induction patterns between IFN- α , IFN- β , and IFN- λ in response to influenza virus and poly(I-C) stimulation of AECs. Although AECs do not secrete IFNs in response to TLR ligands other than poly(I-C), all of the TLR ligands tested here were capable of inducing AEC activation and IL-8 secretion. Thus, TLR-mediated signaling responses are differentially regulated in a cell type- and cytokine-specific manner.

Our study supports the existence of differences in the regulation of type I and type III IFN production in AECs. IFN- λ was the primary IFN produced by hAECs and mTECs, and IFN- λ is also the main IFN secreted by alveolar type II cells (53) and Beas-2B cells (54) and is preferentially induced in the lungs of mice during influenza virus infection (28). Thus, the IFN- λ system is the main cytokine of the IFN family mediating innate defenses in the lung against respiratory viruses. The present study also sheds light on the complexity of IFN- λ regulation. In response to TLR3 stimulation, IFN- λ production was decreased in IFNAR^{-/-} epithelial cells and completely abrogated in Stat1^{-/-} mTECs, a finding in agreement with studies in mice showing that the production of IFN- λ was decreased but not impaired in the absence of IFNAR signaling (28). However, in response to influenza virus infection, IFNAR^{-/-} mTECs produced more IFN- β and IFN- λ than wild-type cultures, indicating the existence of compensatory and cross talk mechanisms between type I and type III IFNs. Clearly, these results demonstrate that a disrupted IFNAR system does not lead to impaired production of IFN- λ , as has been previously suggested (55). Since IFN- λ production was observed in Stat1^{-/-} mTECs at levels similar to those in wild-type cultures in response to influenza virus infection but not in response to poly(I-C) stimulation, it is apparent that the existence of a novel STAT1-independent mechanism of IFN- λ induction is likely to occur downstream of cytoplasmic viral sensors but not downstream of TLR3. Further experiments are required to comprehensively investigate the regulatory mechanisms governing the expression of type III IFNs in human and mouse airway epithelia. A comparison of the human and murine AEC responses to the influenza viruses Udorn

and WSN, respectively, found numerous similarities in the IFN, IFN-inducible gene, and pathogen recognition receptor pathway responses (29). Nevertheless, distinct virus strains have different tropisms that likely affect the innate responses of the airway epithelia. Of the two influenza virus strains used here, the H3N2 human Udorn strain can use α 2,3- and α 2,6-linked sialic acid receptors, while the mouse-adapted H1N1 WSN strain only infects cells expressing the α 2,3-type receptor (56). In addition, sialic acid receptor distribution is epithelial cell specific and species specific (56). These are important differences that need to be considered in understanding the epithelial response to infection.

The role of TLR3 during infection of the respiratory tract is still controversial. TLR3 mediates an inflammatory and antiviral response that is damaging for host survival during influenza virus infection (36, 52, 57). Remarkably, TLR3 has a critical but unexplained role in protection during nonviral infections of the lung, including *Haemophilus influenzae* (58), *Francisella tularensis* (59), and *Schistosoma mansoni* (60) infections. In the complete absence of TLR3, we could not detect any IFN- λ production after stimulation with poly(I-C), which demonstrates the essential role of TLR3 for the detection of naked dsRNA by AECs. Nevertheless, bafilomycin treatment also inhibited a TLR3 response to poly(I-C), indicating that endosomal internalization of cell surface TLR3 is critical for the response of epithelial cells to poly(I-C) and the subsequent IFN- λ induction. TLR3 expressed on intestinal epithelial cells can act as a necrosis sensor during peritonitis and gut inflammation (61). Thus, it is possible that TLR3 expressed on the apical and basal cell surfaces of AECs acts as a sensor of tissue damage by detecting endogenous cellular RNAs (62, 63) or viral dsRNA replication intermediates released by necrotic cells. Regardless of the origin of the ligands, it is likely that the strong inflammatory properties of TLR3 in AECs (51, 64) and its localization at the cell surface contribute to virus-induced exacerbations of respiratory diseases such as COPD or asthma.

In conclusion, our study shows that AECs express TLRs with a distribution that is specific and polarized, that TLR-mediated responses are differentially regulated in a cell type- and cytokine-specific manner, and that type I and type III IFNs have differential induction and regulation patterns in AECs.

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